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GRANT NUMBER DAMD17-96-1-6015

TITLE: Actions and Substrates for the HER4 Tyrosine Kinase in
Breast Cancer

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REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

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Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 1999	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 98 - 30 Jun 99)	
4. TITLE AND SUBTITLE Actions and Substrates for the HER4 Tyrosine Kinase in Breast Cancer			5. FUNDING NUMBERS DAMD17-96-1-6015	
6. AUTHOR(S) H. Shelton Earp, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of North Carolina at Chapel Hill Chapel Hill, North Carolina 27599-1350			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) Please see attached.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 17	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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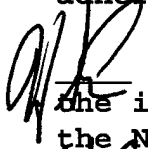
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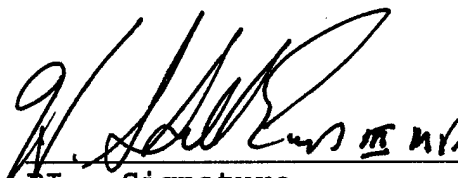

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Report for DAMD 17-96-1-6015
Abstract

During the third year of our HER4 grant, we have made progress in developing methods and cell lines to study the potentially unique role of HER4 in breast cell differentiation. These reagents will help us elucidate the potential oncogenic or anti-oncogenic role of HER4 signaling pathways. We have examined breast cancer cell lines that do or do not express HER4 and have demonstrated in at least one that heregulin-dependent HER4 activation results in an anti-proliferative response. We have determined that these cells, under the influence of heregulin increase their production of neutral lipids, a marker of differentiation. Cell cycle analysis demonstrated that the differentiated cells had a greater than 2N DNA content indicating a G2/1M arrest or endoreduplication. In addition, we created a new cell line by infecting a retroviral versus expressing HER4 and the neo-gene, we then selected cell lines which expressed HER4. This model also exhibited HER4-dependent differentiation. We have devised a flow cytometry assay for assessing the production of neutral lipids as a marker of breast cell differentiation and have begun the task of creating HER4 monoclonal antibodies.

Introduction

The human EGF receptor 2 gene (HER2) is amplified and overexpressed in 20-30% of invasive breast cancer (1). Moreover, numerous articles indicate that poor prognosis breast cancers exhibit increases in EGF receptor content and/or an EGF receptor autocrine loop with the production of TGF α (2,3). Both EGF receptor and HER2 can interact with each other, or with HER3 leading to growth and proliferation (4,5). However, the 4th member of the family, HER4, in preliminary studies appears to have a different output, differentiation rather than proliferation (6,7). Thus, the HER4 signal may slow the growth of breast cancer. Our tasks are to obtain definitive evidence that HER4 provides a different biologic signal in breast epithelium, i.e., differentiation rather than proliferation, and to elucidate the pathway, or elements of the pathway, that differ between HER4 and the original three members of this receptor family (EGF receptor, HER2 and HER3). To this end, we are creating molecular reagents, cell lines and devising new technology which will allow us to prove that HER4 sends a differentiation signal. These reagents should also give us the wherewithal to isolate the unique members of the HER4 pathway as well as substrates phosphorylated by this tyrosine kinase.

Body

In year three, we have concentrated on task one devising, and then studying breast cancer cell models distinguishing the growth promoting action of the EGF receptor versus the differentiating promoting effect of the HER4. This has created systems for us to take on the Task 2, the definition of intercellular pathways activated by HER4 signaling and Task 4, identifying novel HER4 substrates.

A. Studies of the SUM 44 Cell Line.

We obtained a number of breast cancer cell lines, including those from Steve Ethier at the University of Michigan. Figure 1 shows that treatment of these various cell lines with heregulin resulted in growth suppression in the SUM 44 cell line. Figure 2 shows the

anti-proliferative response to heregulin and HB-EGF, both of which are capable of activating HER4. Figure 3 shows the extent of HER4 tyrosine phosphorylation in SUM 44 cells treated with heregulin or HB-EGF. It is clear that heregulin stimulates a HER4 signal as a judge by tyrosine phosphorylation to a greater extent than HB-EGF. These experiments were accomplished using our polyclonal HER4 antisera that was created and tested during year 2. This antiserum is excellent for immunoprecipitation and western blotting and, we believe, is superior to any commercial antibody on the market. Figure 5 shows that som44 cells treated with heregulin elaborate neutral lipids indicating that the tyrosine phosphorylation of HER4 results in a true differentiation phenotype. Figure 6 shows a fluorescence activated cell sorter analysis of som44 cells treated with heregulin. This demonstrates that cells enlarge as they differentiate, and when one examines the DNA content of the cells in the gate with enlarged cells, there is a greater than 2N DNA content. This could be interpreted as cell cycle arrest at the G2M border, but it could also be a result of polypliod development in differentiated breast cell lines. We will investigate this during the next year.

B. Development of a Matched Pair of Cells Expressing or not Expressing HER4.

We decided for sophisticated molecular studies of HER4 signaling that we needed a matched pair of cells which expressed or did not express HER4. We screened available cell lines and determined that the som102 cell line did not express the HER4 receptor. Our molecular constructs of HER4, made in years 1 and 2, were cloned into a retroviral vector. This vector was packaged as an amphotrophic retrovirus and used to infect hard to transfect cell lines such as the SUM 102 cell. G418 selection resulted in the creation of two types of cells, SUM 102-HER4 and SUM 102 neovector. Figure 7 provides convincing evidence in SUM 102 neovector cells did not contain HER4 activated by heregulin. On the other hand, several clones were obtained in which heregulin stimulated tyrosine autophosphorylation. Thus, we have created a matched pair of cell lines expressing HER4 or vector alone. These cells have been tested for growth inhibition response. Figure 8 demonstrates an anti-proliferative response of HER4 containing cells to heregulin and a somewhat diminished growth inhibitory response to HB-EGF. Figure 9 shows the results of a new assay we have developed. The cells treated with or without heregulin were stained with Nile Red and then analyzed by FACS. Heregulin produced a significant shift in Nile Red positive cells providing evidence that not only did heregulin slow the growth of the cells expressing HER4, but heregulin resulted in differentiation. The control SUM 102 neo vector cells did not respond to heregulin.

C. Creation of a Monoclonal Antibody

We used and purified the extracellular domain of HER4 cloned into a baculoviral vector (year 2). This was used through a contract with North Carolina State University to immunize mice. Polyclonal mouse antisera were obtained from two mice, and at least one resulted in the detection of HER4 in an ELISA reaction and was capable of blotting HER4 from cell lysates. Splenic fusion has taken place and we have begun to screen the supernatants from over 800 wells. We have selected 36 wells to be grown up and are awaiting positive supernatants for further screening. Our intention is to select a monoclonal HER4 antibody that will not only recognize HER4 extracellular domain, but will be useful for recognizing HER4 in paraffin-embedded, formalin fixed tissues. This would enable us to assess HER4 expression in a number of archival breast cancer

samples. In this way we could correlate HER4 expression and HER2 expression. Then we could determine whether HER4 modified the generally poor prognosis seen in HER2 overexpressing breast cancers and ask whether there is inverse correlation between HER2 and HER4 expression.

D. Key Research Accomplishments.

- Cloning of full length HER4 cDNA
- Cloning of chimeric EGF receptor HER4 cDNA's
- Site directed mutagenesis of above 4cDNAs to abolish tyrosine kinase activity
- Creation of Retroviral vectors expressing HER4 and dominant negative HER4
- Creation of polyclonal antisera against the C terminus of human HER4
- Creation of an extracellular domain, His-tagged baculovirus expressing HER4
- Immunization of mice and beginning to screen for HER4 monoclonal antibodies
- Creation of 32D cell lines expressing the EGF receptor HER4 chimera
- Demonstration that the HER4 chimera 32D line slows growth in response to ligand
- Demonstration of heregulin-dependent HER4 tyrosine phosphorylation in some breast cancer cell lines
- Demonstration that HER4 signaling results in breast cell line differentiation
- Creation of matched pair of cell lines expressing vector or full length HER4
- Demonstration that HER4 signaling in cell lines expressing HER4 causes differentiation
- Creation of a new flow cytometry assay to assess neutral lipids in differentiating breast cell lines
- In collaboration with David Lee, attempts at creation of transgenic animals expressing EGF receptor HER4 chimera

Section 8-Outcomes

We presented a poster at the NCI SPORE in July of 1999 describing the HER4 differentiating effects noted in our progress report.

Section 9-Conclusions

We are developing a body of evidence that indicates that HER4 signaling does indeed send a different message (anti-proliferation) than other members of the EGF receptor family. This has been demonstrated in an indicator cell line and in breast cell lines. We have shown that a HER4 signal slows growth in two breast cancer cell models, the som44 and the som102 and we have used these two models (naturally occurring HER4 expressing cell or matched pairs of cell lines that we have created) to demonstrate that HER4 causes differentiation in a heregulin dependent manner.

We have created numerous reagents in the study of HER4 including clones and polyclonal antisera. We are in the midst of preparation of a monoclonal antibody that we hope will allow us to perform translational studies using archival-paraffin embedded, formalin-fixed sections. Cell lines, particularly the matched pair of vector and HER4 expressing cells will allow us in the next year to study tyrosine phosphorylated

substrates in a controlled genetic background and will enable us to look at the downstream pathways that may transmit anti-proliferative and cell differentiation signals.

Response of breast cancer cell lines to heregulin stimulation

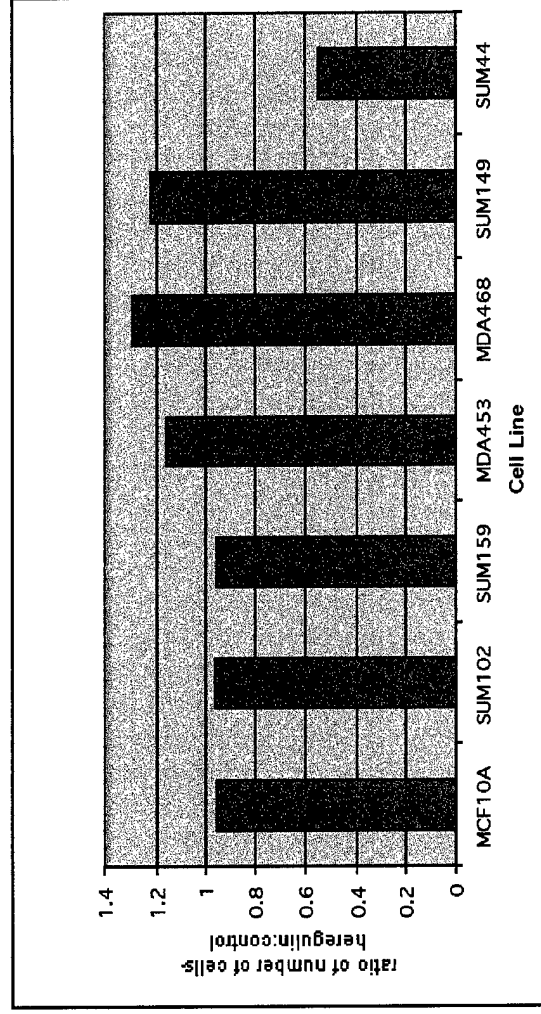


Figure 1. Cells from several breast cancer cell lines were grown in the presence or absence of 10ng/ml of heregulin B1 for 2-3 feedings (4-6 days). At near-confluence, cells were trypsinized and counted. The ratio of number of cells treated with heregulin compared to control is shown (average of at least 3 experiments). Heregulin caused a reproducible anti-proliferative effect in SUM-44 cells, inhibiting growth by 43%, comparable to the degree of inhibition seen with TGF-B.

Anti-proliferative response to a HER-4 specific ligand

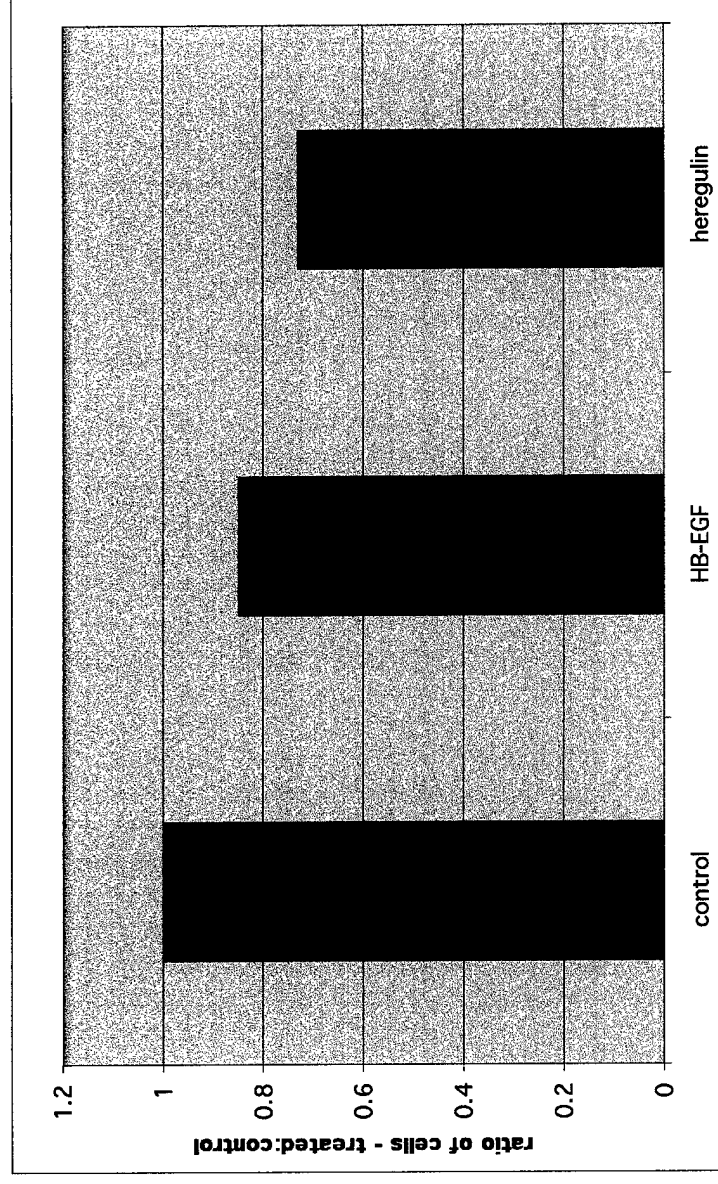


Figure 2. Since heregulin can activate signaling through HER-2 and HER-3, we looked at the effect of a HER-4 specific ligand. Since SUM-44 cells are EGFR negative, HB-EGF binds exclusively to HER-4 in these cells. SUM-44 cells were grown in the presence or absence of 10ng/ml of heregulin B1 for 2-3 feedings (4-6 days). At near-confluence, cells were trypsinized and counted. The ratio of number of cells treated with heregulin compared to control is shown (average of at least 3 experiments). HB-EGF causes decreased proliferation, not as significant as heregulin.

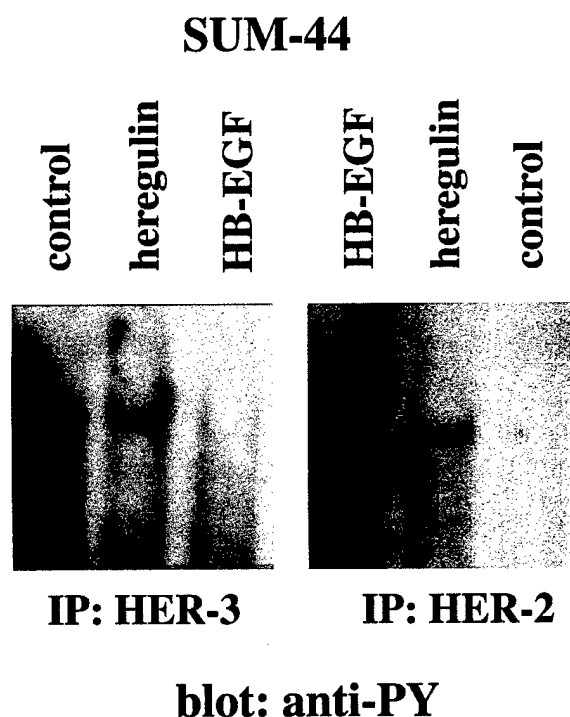
HER-4 Activation in SUM-44 Cells by Heregulin and HB-EGF

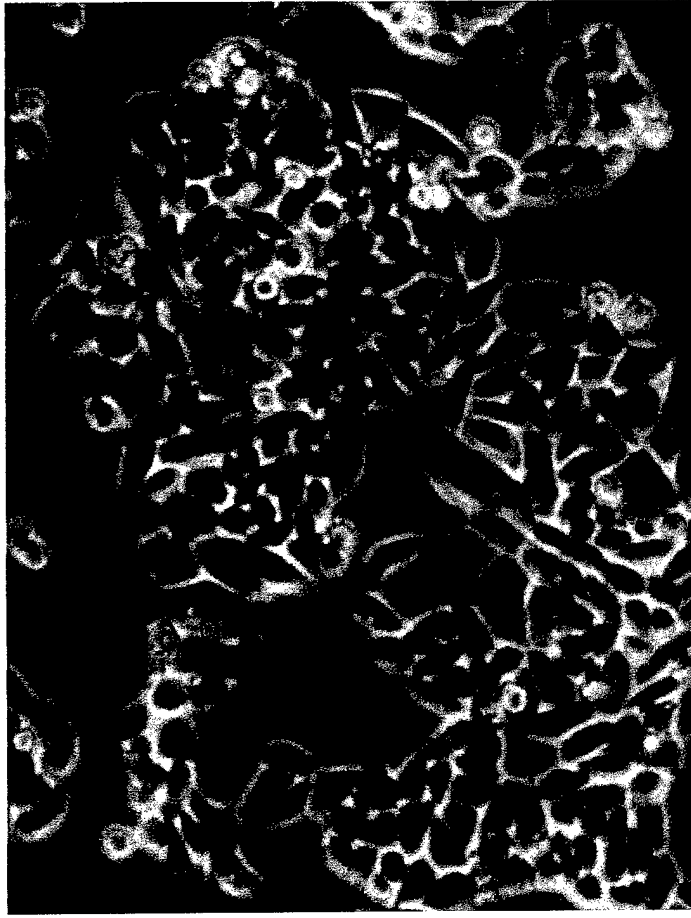
Figure 3. SUM-44 cells were treated with 10ng/ml heregulin B1 or 100ng/ml HB-EGF, lysed, and total protein immunoprecipitated with 5ul HER-4 antiserum. Immunoprecipitates were subjected to PAGE and blotted with mouse monoclonal anti-phosphotyrosine PT66. SUM-44 cells have no tyrosine phosphorylated HER-4 under control conditions, but heregulin and HB-EGF induce HER-4 tyrosine phosphorylation, HB-EGF to a lesser extent than heregulin.



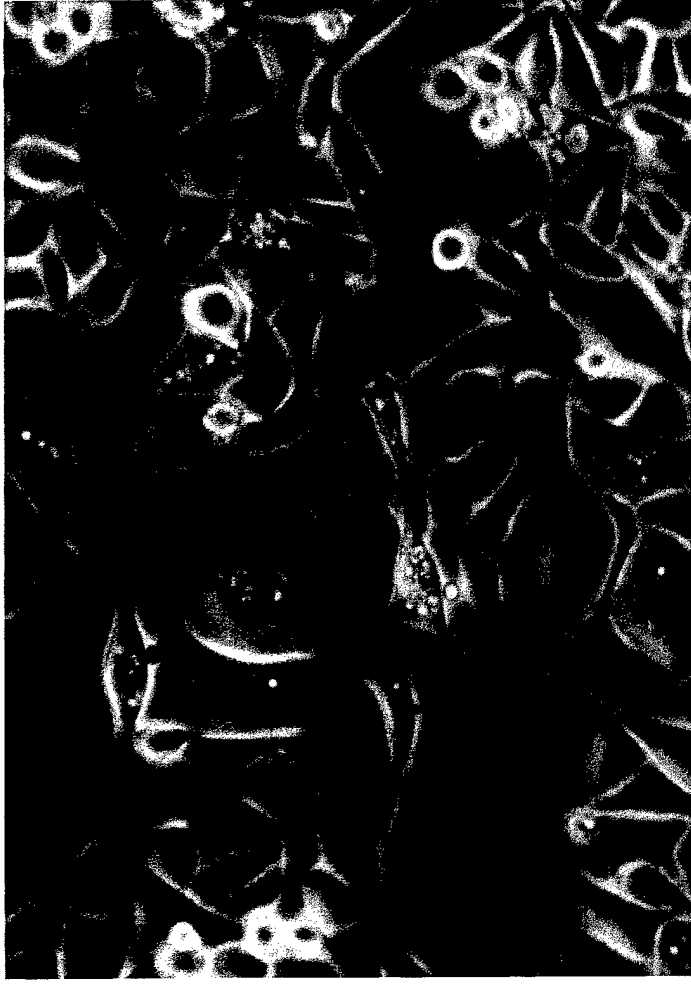
HER-2 and HER-3 Activation by Heregulin and HB-EGF

Figure 4. To determine whether other EGFR family members are activated in SUM-44 cells by ligands that activate HER-4, cell lysates were immunoprecipitated with HER-2 or HER-3, and blotted with anti-phosphotyrosine. Heregulin induces tyrosine phosphorylation of HER-2 and HER-3, while HB-EGF does not. HER-3 is constitutively activated.





SUM-44 control



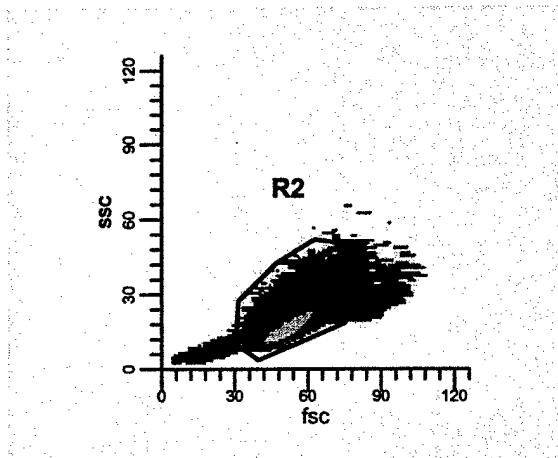
SUM-44 heregulin

Figure 5. To determine whether heregulin and HB-EGF were inducing differentiation changes, cells were grown on glass coverslips for 4-6 days in the presence of heregulin or HB-EGF, fixed in formamide, and stained with Sudan IV to detect neutral lipids. Heregulin and HB-EGF (not shown) caused cell fattening and accumulation of droplets that contain lipids produced during milk protein synthesis, indicating differentiation.

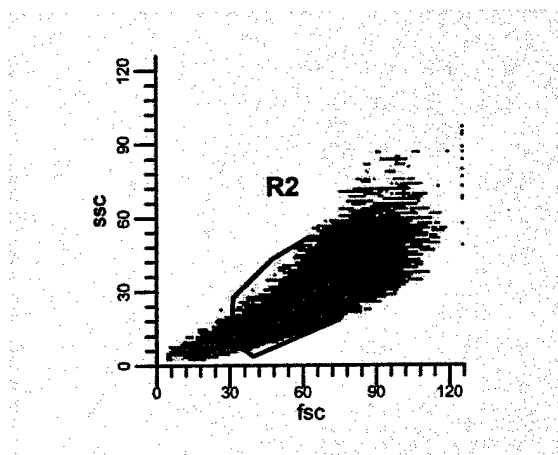


Sudan IV stain

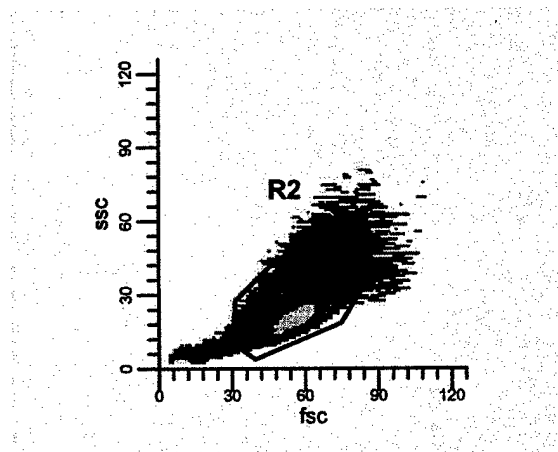
HER-4 Activated Cells Exhibit 4N or Greater DNA Content



control



heregulin



HB-EGF

Figure 6. To determine the effect of HER-4 activation on cell cycle, SUM-44 cells were grown in the presence of heregulin or HB-EGF for 4-6 days, fixed in ethanol, and cell cycle was analyzed by flow cytometry. In the presence of heregulin or HB-EGF, there was a shift to higher DNA content and greater granularity and cell size. Cell cycle analysis showed that a large proportion of the cells in the shifted portion of the profile have 4N or greater content, compared to the unshifted population, particularly in the heregulin treated cells.

	<u>G0/G1</u>	<u>S</u>	<u>% 4N DNA</u>
control	49%	11%	36%
HB-EGF			
- cont. gate	36%	20%	43%
- shift	32%	16%	51%
Heregulin			
- cont. gate	54%	17%	28%
- shift	18%	11%	70%

Stably Infected SUM-102 Cells Express HER-4 that is Activated by Heregulin

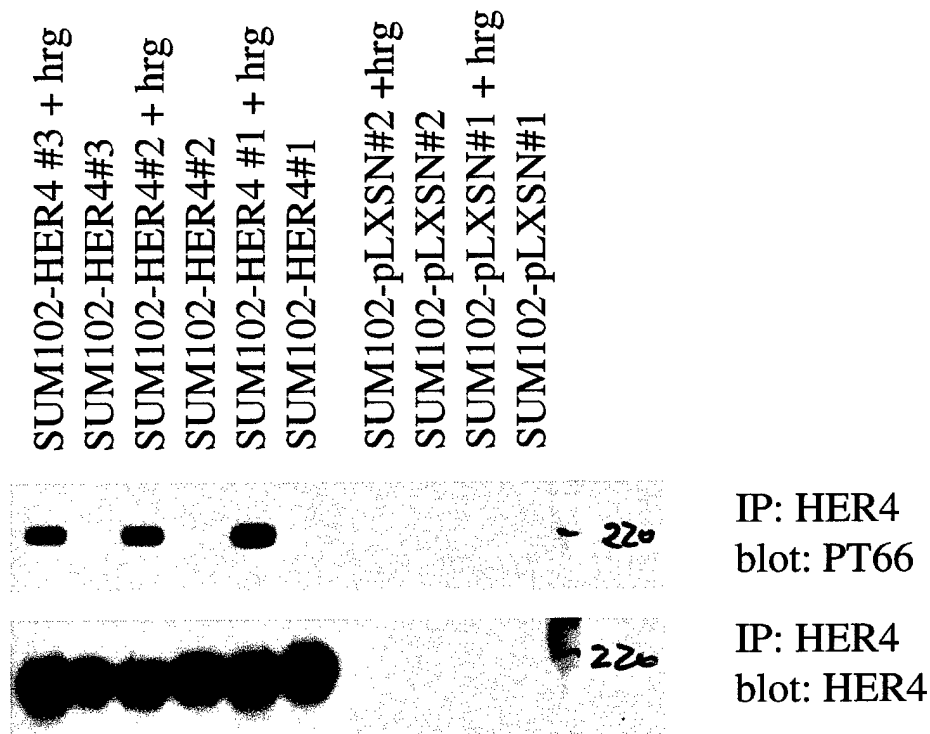


Figure 7. Full-length HER-4 was stably expressed in SUM-102 cells, a human breast cancer cell line that is HER-4 negative, by retroviral infection and selection for G418 resistance. HER-4 expression was confirmed by Western blot using HER-4 anti-serum. Vector expression was confirmed in control cells by PCR (data not shown). Tyrosine phosphorylation of HER-4 in response to heregulin stimulation was measured by immunoprecipitation and western blotting as described. SUM-102 cells. In SUM-102/HER4 lines, HER-4 is not constitutively activated, but is activated in response to ligand stimulation.

SUM102-HER4 Cells are Growth-Inhibited by HER-4 Ligands

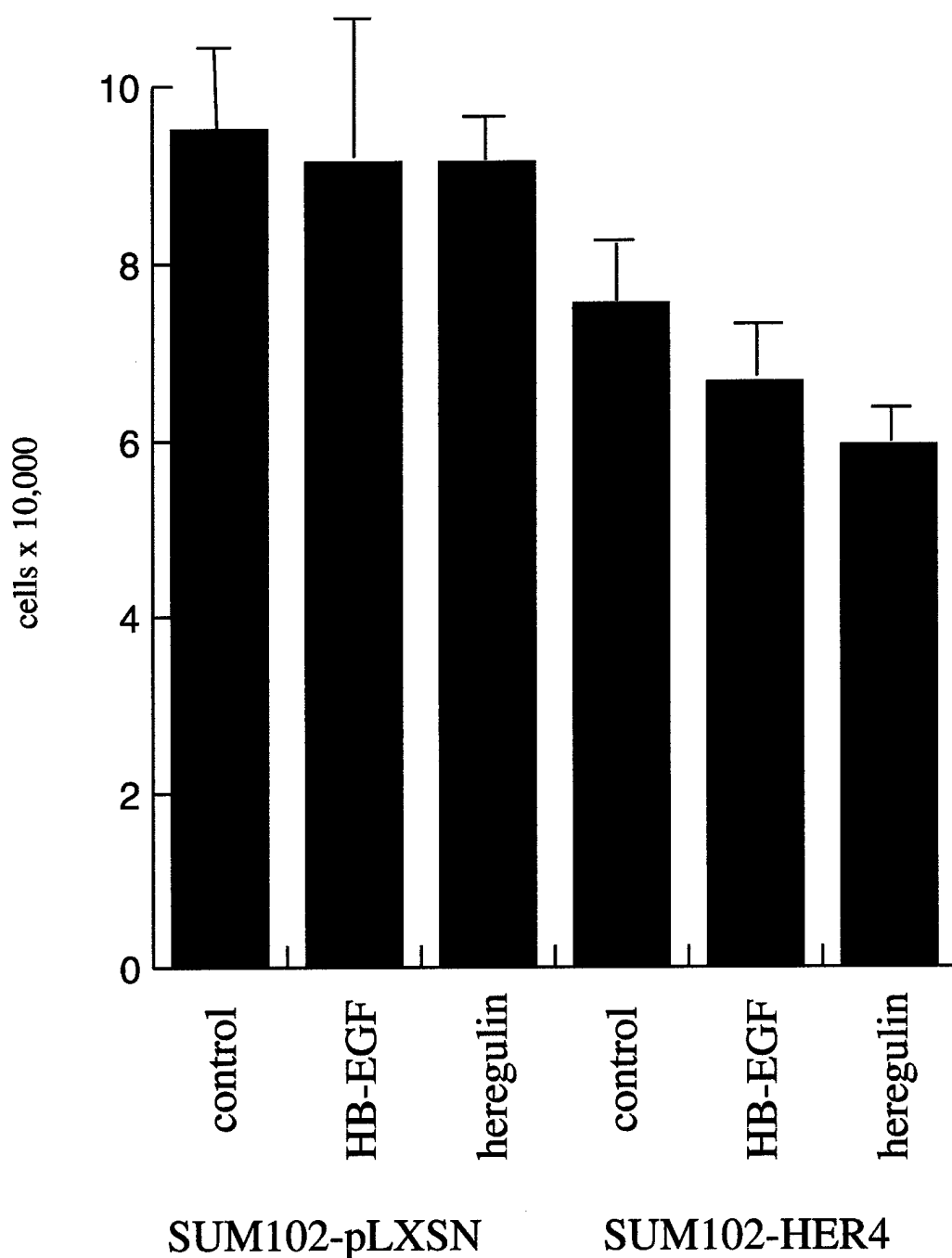
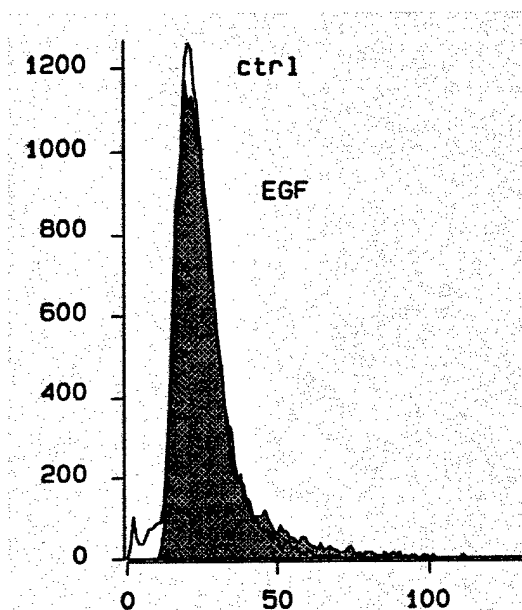
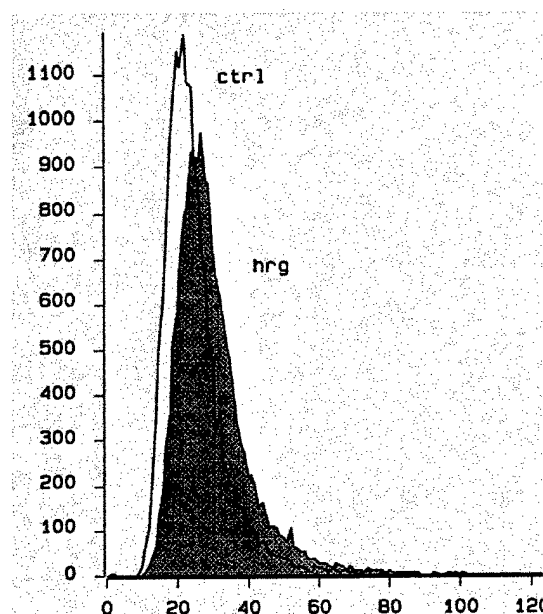


Figure 8. To determine whether expression of HER-4 in SUM-102 cells causes the cells to acquire a response to HER-4 ligands, cells were grown in the presence or absence HB-EGF (100ng/ml) or heregulin (10ng/ml) for 4-6 days. SUM102-HER4 cells are growth inhibited with HB-EGF and heregulin, comparable to SUM-44 cells, while wild-type and vector control SUM-102 cells do not have an antiproliferative response to HER-4 ligands.

SUM-44/HER4 Cells Treated with Heregulin Produce Neutral Lipids

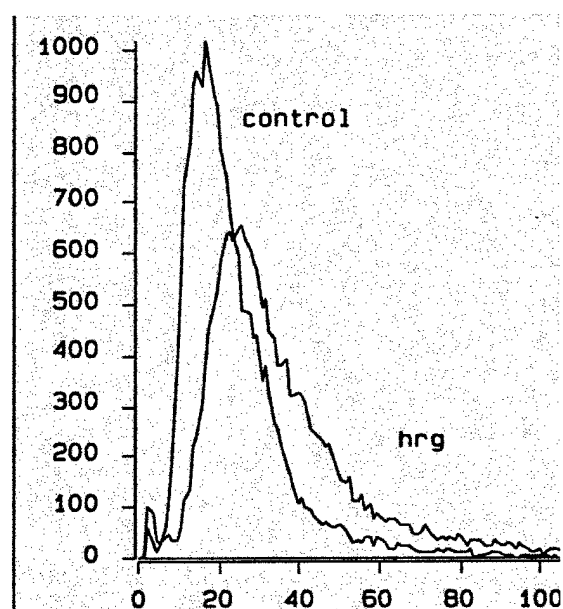


SUM-102/pLXSN



SUM-102/HER4

Figure 9. SUM-102 cells expressing vector or HER4 were treated with 10ng/ml heregulin for 4-6 days, and stained with Nile Red. Intensity of staining was measured by flow cytometry, and histograms of control and heregulin-treated cells overlaid. SUM-102/HER4 cells have increased neutral lipid staining when treated with heregulin, comparable to SUM-44 cells, while HER4 negative control cells do not.



SUM-44

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